Moniliformin Analysis in Maize Samples from North-West Italy Using Multifunctional Clean-up Columns and the LC-MS/MS Detection Method.

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Moniliformin Analysis in Maize Samples from North-West Italy Using Multifunctional Clean-up Columns and the LC-MS/MS Detection Method

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ABSTRACT: A fast clean-up method has been developed to purify maize extracts and to detect moniliformin (MON) in maize samples from North-West Italy over a four-year period (2008-2011). The method is based on the use of MycoSep® 240 Mon clean-up columns (Romer Labs®). Samples were extracted using acetonitrile/water (84:16, v/v), and the extracts were purified in the previously described clean-up columns. The LC-MS/MS analysis has been carried out by means of hydrophilic interaction chromatography (HILIC), combined with negative electrospray mass spectrometry (ESI^- - MS). The developed method has a recovery rate of 76-91% (RSD%: 6-14%), a limit of detection (LOD) of 1 μg kg^{-1}, and a limit of quantification (LOQ) of 4 μg kg^{-1}. One hundred and eight different naturally contaminated maize samples were analyzed for their MON content. The average percentages of positive samples was 93% with the following ranges (μg kg^{-1}): 33-2606 (2008); < LOD-527 (2009); < LOD-920 (2010); < LOD-409 (2011).

KEYWORDS: Moniliformin, maize, mycosep clean-up columns, LC-MS/MS, *Fusarium*

ABBREVIATIONS

EFSA, European Food Safety Authority; ESI, electrospray ionization; GDD Accumulated growing degree days; HILIC, hydrophilic interaction chromatography; HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MON, moniliformin; MS, mass spectrometry detection; RSD, relative standard deviation; SAX, strong ion exchange; SPE, solid phase extraction.

INTRODUCTION

Moniliformin (MON) is a worldwide *Fusarium* mycotoxin which often occurs in cereals and maize (Sharman *et al.* 1991). MON is a small (98.0081 g mol^{-1}) (Betina 1989), highly polar, acidic molecule. Due to the low pK_a value (< 1.7) of the free acid (semisquaric acid), MON does not occur as an acid in nature but as a water soluble sodium or potassium salt (Steyn *et al.* 1978).

It was first isolated by Cole et al. in 1973 (Cole *et al.* 1973) and structurally characterized as the sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione (Figure 1) by Springer et al. in 1974 (Springer *et al.* 1974). MON can produce plant growth regulation and phytotoxic effects in plant systems (Cole *et al.* 1973, Vesonder *et al.* 1992). MON is also toxic to several animal species, causing myocardial changes, muscular weakness, respiratory distress, cyanosis, coma, and death (Kriek *et al.* 1977). The action mechanism probably involves selective

22 inhibition of the pyruvate and α-ketoglutarate dehydrogenase enzyme systems
23 (Burka *et al.* 1982). MON toxicity mainly affects cockerels (LD_{50} = 4.00 mg kg^{-1},
24 oral) and ducklings (LD_{50} = 3.68 mg kg^{-1}, oral) (Kriek *et al.* 1977).
25 MON is produced by several *Fusarium* species on several crops, and has been
26 found in different geographical areas. The main *Fusarium* species that are able to
27 produce MON are listed in Table 1. MON contamination is higher in maize than in
28 other substrates and, in South Europe, it is commonly produced in maize infected
29 by *F. proliferatum* and *F. subglutinans*. Both species can be found globally, but the
30 optimum temperature for growth of *F. subglutinans* is lower than that of *F.*
31 *proliferatum*, thus the former is more common in temperate areas (Kostecki *et al.*
32 1999). In a study of the incidence, geographic distribution and toxigenicity of
33 *Fusarium* species in South African maize, *F. subglutinans* was found to
34 predominate in relatively cool and humid climates (Rabie *et al.* 1982). Since 1982,
35 when it was first reported as a natural contaminant in Transkeian maize (16-25 mg
36 kg^{-1}) (Thiel *et al.* 1982), MON has been found in maize in different parts of the
37 world. This mycotoxin was detected in maize ears in Poland, from 1985 to 1991,
38 and the average content over these six years was 131 mg kg^{-1} (Chelkowski *et al.*
39 1987, Chelkowski 1989, Lew *et al.* 1996). It has also been found to occur in
40 Austrian maize, with levels of up to 20 mg kg^{-1} (Lew *et al.* 1991), as well as in
41 Canada, Germany, and New Zealand (Lamprecht *et al.* 1986, Scott *et al.* 1987,
42 Thalman *et al.* 1985). In these surveys the original samples were hand-selected in
43 order to collect visibly *Fusarium* infected kernels, thus high levels of MON

contamination were reported. Instead, the results of surveys on MON concentrations and distribution in naturally contaminated maize grain from field or commercial lots, which are reported in Table 2, have shown lower levels of MON concentration.

Several approaches have been developed for the selective extraction, sample purification, chromatographic separation and detection of MON.

Liquid Chromatography has been the main chromatographic technique used in the analysis of MON and due to the highly polar properties of this mycotoxin the most common procedure used in the years was the application of ion-pairing mobile phases to achieve a good chromatographic separation with reversed-phase columns (Shepherd and Gilbert 1986, Munimbazi and Bullerman 1998, Sewram *et al.* 1999). More recent approaches to improve the chromatographic separation were the use of hydrophilic interaction chromatography (HILIC) (Sørensen *et al.* 2007) and the Gemini C6-Phenyl column as reversed-phase column (Von Bargen *et al.* 2012).

Until now, the several analytical methods that have been reported in literature have used commercial SPE-columns for the purification of MON extracts. These mainly have been strong anion exchanger (SAX) columns (Munimbazi and Bullerman 1998, Filek and Lindern 1996, Parich *et al.* 2003, Sørensen *et al.* 2007, Von Bargen *et al.* 2012), but also nonpolar C_{18}-columns (Shepherd and Gilbert 1986), or a combination of the two (Sharman *et al.* 1991). However, Jestoi et al. (Jestoi *et al.*

2003) did not apply any sample purification steps, except filtering and concentration, in order to reduce the loss of MON during the analysis.

Since these purification methods are time consuming and require several steps, they could lead to analytical errors and a detriment of analytical repeatability. In addition, not applying a purification step can lead to the instrumentation becoming dirty, thus impairing the analysis.

The purpose of the current study was to develop a fast, reliable and repeatable clean-up method, using MycoSep® 240 Mon clean-up columns (Romer Labs®) for the first time to purify maize extracts prior to the analytical determination of MON. Moreover the European Food Safety Authority (EFSA) is currently working on establishing a scientific opinion on the risks to public health related to the presence of MON in feeds and food (EFSA 2010). Since there is this need to obtain major information about the incidence of this mycotoxin in the most important cereal areas in the EU, this procedure has been applied to maize samples collected over a four-year period (2008-2011) in North-West Italy in order to obtain data about the presence, diffusion and level of MON contamination. A first attempt has been made to individuate the conditions which could lead to a higher contamination of this mycotoxin.

MATERIALS AND METHODS

Chemicals

All the chemicals and analytical standards were purchased from Sigma Aldrich (St. Louis, MO), or VWR (Milan, Italy). The solvents were gradient grade or LC-MS grade.

The MON standard was purchased as sodium salt and a 93 mg L^{-1} stock solution of MON in acetonitrile/water (84:16, v/v) was prepared and stored at 4 °C. This stock solution was used to prepare standard solutions through dilution with acetonitrile/water (84:16, v/v).

Samples

One hundred and eight maize grain samples, collected over 4 years in farm fields in North-West Italy (Torino and the Cuneo Province), were analyzed for natural MON contamination. The number of maize grain samples collected each year was 16, 16, 40 and 36 in 2008, 2009, 2010 and 2011, respectively. The fields were cultivated under irrigation with full length maturity hybrids, planted in each growing season in the period between the last decade of March and the first decade of April. The normal agronomic techniques of each area were adopted. The considered fields were characterized by a natural infestation of European Maize Borer (ECB, *Ostrinia nubilalis* Hübner) each year and in each site, since no insecticide was applied to control ECB or other insects during the ripening period.

One hundred ears (included the ears used for the evaluation of ECB incidence and severity at harvest) were collected by hand for each maize kernel sample in each field at the end of maturity (moisture content of grains of between 23 and 27%) from five subplots and shelled using an electric sheller. The kernels from each plot were mixed thoroughly to obtain a random distribution; 5 kg samples were then taken to analyze the MON content and dried at 50°C for 3 days.

ECB damage incidence was calculated as the percentage of ears per plot with kernel injuries or apical and basal tunnels in the cob due to larvae activity. The ECB damage severity was calculated as the percentage of kernels per ear with injuries due to larvae activity. A scale of 1 to 7 was used in which each numerical value corresponded to a percentage interval of surfaces exhibiting visible kernel damage due to larvae activity according to the following schedule: 1 = no injuries, 2 = 1-5%, 3 = 6-10%; 4 = 11-20 %, 5 = 21-35%, 6 = 35-60%, 7 > 60% (Blandino *et al.* 2009). The ECB damage severity scores were converted to percentages of ears exhibiting symptoms and each score was replaced with the mid-point of the interval.

Chemical Analyses

Sample Preparation and Extraction

Maize samples were ground using a ZM 200 Ultra Centrifugal Mill (Retsch GmbH, Haan, Germany) and the flour was used directly for the extraction. Twenty five g ground maize samples from a MON-free sample were spiked in order to evaluate the recovery rate of the analytical method. Three replicas with 100 µL of pure solvent, or 0.93; 9.3; 93 mg MON L^{-1}; or 1000 µL of 93 mg MON L^{-1} were used to obtain spiked MON levels of 0, 3.72, 37.2, 372, and 3720 µg kg^{-1} for LC-MS/MS determination. The experiments were performed on four different days to establish day to day variations. The spiked maize samples were incubated at room temperature 2 h prior to extraction, which allowed the solvent to evaporate and MON to enter the material.

Twenty five g maize flour was extracted by mechanical shaking at 100 rpm for 1 h (shaker mod. M102-OS, MPM Instruments, Milan, Italy) with 100 mL acetonitrile/water (84:16, v/v). The extracts were filtered through Whatman no. 1 filters (Brentford, UK) and subjected to clean-up and purification.

Clean-up

Two clean-up methods were tested. The first method was performed with Strata SAX (Strong Anion Exchange) columns (500 mg) (Phenomenex, Torrance, CA) applying the clean-up procedure described by Sørensen et al. (Sørensen *et al.*

2007). The columns were activated with 2 mL methanol, 2 mL water, and 2 mL 0.1 M HCl. Two mL of concentrated maize extracts were then added to the SAX columns. The cartridge was washed with 2 mL methanol-water (50:50) and 2 mL 0.1 M HCl. MON was eluted with 2 mL 1 M HCl. The eluate was dried under nitrogen, diluted in 100 μL acetonitrile-water (85:15) and transferred to an HPLC vial. The second clean-up method was performed with MycoSep® 240 Mon clean-up columns (Romer Labs®, Tulln, Austria). The clean-up procedure was adapted from the Romer Labs® procedure. The cleanup MycoSep® columns were pushed into test tubes containing 5 mL of the sample extracts, forcing the extracts to filter upwards through the packing material of the columns. The interferences adhered to the chemical packing in the columns and the purified extracts, containing MON, passed through the columns. The evaporation to dryness under nitrogen step was excluded because it provoked a loss of MON of up to 40%. The use of silanized vials, which were adopted to avoid the adsorption of MON on the glass, did not improve the recovery. The purified extracts (1.5 mL) were transferred to HPLC vials and analyzed by means of LC-MS/MS according to the method described below.

This one step cleanup required less than one minute per sample, while the method based on SAX clean-up, because of the long time required to evaporate the HCl aqueous solution, was much more time consuming.

LC-MS/MS

LC-MS/MS analysis was carried out on a Varian 310 triple quadrupole mass spectrometer (Varian, Italy) equipped with an electrospray ionization ESI source, a 212 LC pump, a ProStar 410 AutoSampler and dedicated software. LC separation was performed on a 100 mm × 2.1 mm i.d., 3.5 µm, 100 Å ZIC®-HILIC (Merck, SeQuant, Milan, Italy) column. The mobile phase consisted of water buffered with 100 mM ammonium formate (pH 6.4) (A) and acetonitrile (B) delivered at 200 µL/min. The gradient was 5 to 50 % A in 7 min. Mass spectrometric analyses were performed in the negative -ion mode. The nebulising gas was N_2 (20 psi), the drying gas was air (250 °C, 25 psi), the needle voltage was -3000 V, the shield voltage was -600 V, the detector voltage was 1250 V, the capillary voltage was -20 V and the collision energy voltage was 12 V. The deprotonated molecule (*m/z* = 97.0) was fragmented to its product ion (*m/z* = 41.0) and used for quantification and identification purposes.

Calibration

Ten different MON concentrations were prepared for calibration in acetonitrile/water (84:16, v/v), between 0.93 and 930 µg L^{-1}. A linear regression was used to obtain the regression curve.

RESULTS AND DISCUSSION

LC-MS/MS Analyses

Previous studies on the chromatographic separation of MON reported the use of different types of columns. The chromatography of the current study was based on the Sørensen et al. (Sørensen *et al.* 2007) method and the authors own observations during the development of the LC-MS/MS method. The MON retention time with the HILIC gradient program was 3.9 min with a runtime of 17 min (Figure 2). This runtime was necessary to elute, with 50% water buffered with 100 mM ammonium formate, stronger retained contaminant compounds than MON. MON is a strong acid and hence produces more negative than positive ions. Negative ion polarity usually generates less background noise than the positive mode, therefore improving sensitivity. ESI was therefore adopted, in negative mode, in the current study using a triple-quadrupole instrument. The presumed MON fragmentation pathway is shown in Figure 3. Unfortunately, in tandem mass spectrometers MON generates only one strong product ion in the collision cell of the instrument. Thus, only one MRM, the fragmentation of *m/z* 97 to *m/z* 41 can be programmed (Jestoi *et al.* 2003). To improve the sensitivity and specificity of the analysis of MON and to avoid the use of the only possible transition in tandem mass spectrometry, a recent approach was the use of a high resolution instrument (Von Bargen *et al.* 2012).

214 **Clean-up**

215 Several surveys have shown the columns most frequently adopted for SPE clean up
216 procedures, to purify maize and cereal grain extracts, are SAX columns (Sharman
217 *et al.* 1991, Munimbazi and Bullerman 1998, Filek and Lindner 1996, Sørensen *et*
218 *al.* 2007). When these clean-up procedures were applied to ours samples, low
219 recoveries were observed (≤ 50%), with one working day being necessary for five
220 samples. The MycoSep® column clean-up was instead much less time consuming
221 (less than 1 min per sample) and the matrix effect was reduced. This phenomenon,
222 known as suppression, is caused by the co-eluting matrix components, which
223 interfere with the ionization of the analyte (Tang and Kebarle 1993, Gilar *et al.*
224 2001).

225 When the whole Romer Labs® procedure was applied low recoveries were
226 obtained. For this reason it was hypothesised that the concentration step by
227 evaporation to dryness under nitrogen could be a significant factor in the loss of
228 MON, even when silanized vials were used. Higher recoveries were obtained by
229 excluding this concentration step from the Romer Labs® procedure.

230 Thus, because of the higher recoveries and reduced time necessary, MycoSep®
231 columns were used and the previously described clean-up procedure was applied to
232 purify maize samples.

233 The percentage of recovery (Table 3) ranged from 76 to 91 % (Relative Standard
234 Deviation, RSD%: 6-14%), independently of the MON concentration.

235 No differences were observed in recovery rate between the three days, thus
236 confirming the repeatability of the method.
237 The limit of detection (LOD) and the limit of quantification (LOQ) were 1 μg kg^{-1}
238 and 4 μg kg^{-1}, respectively.
239 The results attest the accuracy, repeatability and robustness of the method, which
240 has here been applied to maize extracts for the first time.
241
242 **Naturally Contaminated Samples**
243 The developed analytical method was applied to 108 maize samples collected over
244 a four-year period (2008-2011) in North-West Italy, in order to obtain data about
245 the presence, diffusion and level of contamination of MON in the grain maize
246 cultivated in this area.
247 The considered growing seasons showed remarkably different meteorological
248 trends (Table 4). The years 2008 and 2010 were characterized by low growing
249 degree days (GDDs) and high rainfall, in particular during the 2010 growing
250 season, from the early milk stage to the harvest. The year 2009 had ordinary GDDs
251 and rainfall from flowering to the end of ripening. The year 2011 was instead
252 characterized by high GDDs and extremely limited rainfall from the milk stage to
253 the harvest .
254 Table 5 summarized the percentages of positives samples, the arithmetic mean (±
255 RSD) and the range of MON contamination for each sampling year. Overall, the
256 average percentages of positive samples was 93%, with the following ranges (μg

257 kg^{-1}): 33-2606 (2008); < LOD-527 (2009); < LOD-920 (2010); < LOD-409 (2011).
258 The MON concentration means (µg kg^{-1}) (± standard deviation) for each year were:
259 1127 ± 784 (2008); 106 ± 135 (2009); 262 ± 243 (2010); 89 ± 99 (2011).
260 On the basis of the results of the MON contamination it is possible to state that this
261 mycotoxin is diffused through the investigated areas and shows considerably high
262 levels for each sampling year. However, considering that there is a lack of MON
263 contamination diffusion data in literature and a lack of recent data performing to
264 the most important cereal areas in the EU, it has not been possible to conduct an
265 exhaustive comparison. Nevertheless, on the basis of Table 2 it can be seen that
266 ours results are in agreement with the worldwide levels of MON contamination
267 present in naturally contaminated maize commodities.
268 Table 6 summarized the mean data of ECB incidence and severity of ears collected
269 for each sampling year. The samples collected in the year 2008 showed the highest
270 ECB severity, and thus was followed by those harvested in 2010. The ECB
271 pressure was lower in 2009 and 2011.
272 The collected data confirm an important link between MON contamination and
273 ECB activity performing to the damage of maize ears, which was also observed
274 also by Papst et al. (Papst *et al.* 2005) in Germany, in a comparative study on Bt
275 maize and its isogenic counterparts. Moreover, Papst et al. (Papst *et al.* 2005) and
276 Magg et al. (Magg *et al.* 2003) reported a significant correlation between the
277 percentage of ECB damaged ears and the MON concentration in the kernel: the

278 MON content in ears manually infested with ECB larvae was 3 times higher than
279 the control, which has been protected through an insecticide application.
280 In temperate areas, *F. verticillioides* is favoured more by ECB larvae feeding than
281 other *Fusarium* species (Lew *et al.* 1991). In the years with high rainfall and low
282 temperatures, the development of *F. verticillioides* on damaged areas of kernels,
283 caused by second generation ECB, could be less predominant than other fungi,
284 such as *F. subglutinans* and *F. proliferatum*, which commonly cause MON
285 contamination. On the basis of these assumptions, and the different meteorological
286 trends of the considered growing seasons (Table 4), the mean MON concentration
287 data collected for each year could explain why 2008 and 2010 had the highest
288 MON contamination.
289 In conclusion, the obtained results indicate that the developed clean-up procedure,
290 which was here used for the first time, is very fast, highly accurate, repeatable and
291 robust. Since this method allows a reliable quantification of MON in maize
292 samples as low as 4 μg kg^{-1}, it was possible to quantify MON in more than 90% of
293 the analyzed samples. Moreover, the present work provides a first important series
294 of data on natural MON contamination in maize kernels, referring to several
295 growing seasons with different meteorological trends. The first collected data
296 suggest that the risk of MON contamination in the North Italian cropping area
297 increases for growing seasons with higher rainfall and lower temperatures, from the
298 early milk stage to the harvest. Furthermore, an important link exists between
299 MON contamination in kernels and injuries caused by ECB larvae on maize ears.

300 The first MON results obtained in the current study could be very useful for EFSA
301 in order to assess the risks to human and animal health related to this mycotoxin.
302 These data need to be integrated with information from maize, other cereal grains
303 and derived products from the main cropping areas. Thus, the new clean-up
304 procedure could be utilized to quantify MON occurrence in an accurate but rapid
305 and efficient way.
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TABLES

Table 1. Fusarium species most frequently associated with MON production in maize throughout the world.

Fusarium species	Other mycotoxins produced[a]	References
F. proliferatum	FB, BEA, FP	Logrieco *et al.* 1995 - Miller *et al.* 1995
F. verticilliodes	FB, FUS	Leslie *et al.* 1996 - Sanhueza *et al.* 2004
F. subglutinans	BEA, FP	Kostecki *et al.* 1999
F. avenaceum	BEA, ENN, FUS	Morrison *et al.* 2002
F. chlamydosporum	-	CFP/EFSA/CONTAM/2008/01
F. oxysporum	BEA	CFP/EFSA/CONTAM/2008/01
F. tricinctum	BEA	CFP/EFSA/CONTAM/2008/01

[a] BEA = beauvericin; FB = fumonisin B_1, B_2 and B_3; FP = fusaproliferin; FUS = fusarin C; ENN = enniantins.

Table 2. Results of surveys on MON concentrations and distribution in naturally contaminated cereal commodities.

Country	Year	Cereal	Samples (n°)	LOQ[b] (µg kg^{-1})	*n* > LOQ	Min (µg kg^{-1})	Max (µg kg^{-1})	Sampling procedure	Clean-up	References
Germany	na[a]	Maize	58	na	25	na	> 650	na	na	Talman *et al.* 1985
Worldwide	1985 - 1989	Maize	64	50[c]	27	< 50	3160	Field samples of maize from 10 different countries	SAX columns[d]	Sharman *et al.* 1991
Austria	1991 - 1992	Wheat	48	10[c]	29	< 10	880	Wheat from Austrian fields	na	Adler *et al.* 1995
China	na	Maize	104	na	47	52	1116	Maize from Chinese fields	na	Yu *et al.* 1995
Austria	na	Maize	na	20	na	50	2000	Maize from Austrian fields	SAX columns	Filek and Lindner 1996
South Africa	1997	Maize	4	5[c]	2	< 5	17	Maize samples from Transkei	RP-C18 columns[e]	Sewram *et al.* 1999

USA	1998	Maize	100	25[c]	83	< 25	774	Food-grade commercial maize samples	na	Gutema *et al.* 2000
Poland	na	Wheat	10	na	4	na	200	Wheat from 10 private farms	Columns containing Florisil®	Krysinska-Traczyk *et al.* 2001
Austria	na	Maize	na	39[c]	na	160	1030	Maize from Austrian fields	SAX columns	Parich *et al.* 2003
Finland	2001 - 2002	Barley	22	20	22	< 20	750	Cereals from Southern and Central Finland fields	na	Jestoi *et al.* 2004
		Wheat	14	20	10	< 20	810			
		Oats	1	20	1	-	84			
Norway	2000 - 2001	Barley	75	130	53	< 130	380	Cereals from Norwegian fields	SAX columns	Uhlig *et al.* 2004
		Wheat	83	130	76	< 130	950			
		Oats	73	130	38	< 130	210			
Denmark	na	Maize	28	12	0	1	< 12	Whole maize plants	SAX columns	Sørensen *et al.* 2007

[a] Not available; [b] LOQ = Limit of Quantification; [c] LOD = Limit of Detection; [d] SAX = Strong anion exchange; [e] RP-C18 = reversed-phase (C18).

Table 3. Recovery rate of the analytical method at four MON concentration levels.

MON Concentration [a] (µg kg^{-1})	Recovery [b] (%)	RSD [c] (%)
3720	76	6
372	76	8
37.2	82	14
3.72	91	9

[a] Spiked MON levels (µg kg^{-1}); [b] Mean recovery (%) obtained from three replicas for each MON concentration level in three different days; [c] RSD = Relative Standard Deviation.

Table. 4. Total rainfall, rainy days, relative humidity and growing degree days (GDD 10s) from June to October 2008-2011 in a representative site of the investigated maize growing area .

Year	Month	Rainfall (mm)	Rainy days (n°)	GDD 10s[a] (°C d^{-1})
2008	May	121	16	204
	June	95	17	304
	July	63	8	382
	August	52	6	372
	September	57	8	228
	October	30	5	151
	May-October	418	60	1641
2009	May	30	10	292
	June	26	7	341
	July	121	8	391
	August	56	11	404
	September	62	8	273
	October	54	6	163
	May-October	349	50	1864
2010	May	117	12	214
	June	192	11	332
	July	37	8	420
	August	116	11	354
	September	51	12	240
	October	105	9	120
	May-October	618	63	1680
2011	May	42	7	286
	June	104	13	335
	July	59	7	364
	August	9	2	431
	September	24	3	346
	October	19	1	111
	May-October	257	33	1873

[a] Accumulated growing degree days for each month using a 10°C base.

Data refer to the Carmagnola site (44° 50’ N, 7° 40’ E; altitude 245 m).

Table 5. MON Concentrations in maize samples from North-West Italy over a four-year period (2008-2011).

Year	Samples [a, b] (n°)	Mean MON Concentration [c] (µg kg^{-1})	MON Concentration Range (µg kg^{-1})	Positives [e] (%)
2008	16	1127 ± 784	33 - 2606	100
2009	16	106 ± 135	< LOD [d] - 527	81
2010	40	262 ± 243	< LOD - 920	98
2011	36	89 ± 99	< LOD - 409	92

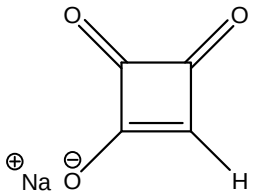
[a] N° Samples = Number of samples analyzed each year; [b] Naturally contaminated maize samples collected in fields in North-West Italy; [c] Concentrations not corrected for recovery; [d] Limit of detection (LOD) = 1 µg kg^{-1}; [e] Positive samples = samples with MON concentration ≥ LOQ.

Table 6. Mean data of ECB incidence and severity of ears collected in each sampling year.

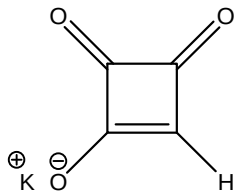
Year	Samples (n°)	ECB incidence[a] (%)	ECB severity[b] (%)
2008	16	99 ± 2	25 ± 4
2009	16	68 ± 19	8 ± 4
2010	40	78 ± 16	13 ± 6
2011	36	40 ± 13	6 ± 2

[a] ECB incidence was calculated as the percentage of ears with ECB damage, considering 100 ears per sample; [b] ECB severity was calculated as the percentage of kernels per ear with ECB damage, considering 100 ears per sample.

FIGURES



sodium 3,4-dioxocyclobut-1-enolate



potassium 3,4-dioxocyclobut-1-enolate

Figure 1. Sodium and potassium salt structures of of 1-hydroxycyclobut-1-ene-3,4-dione (MON).

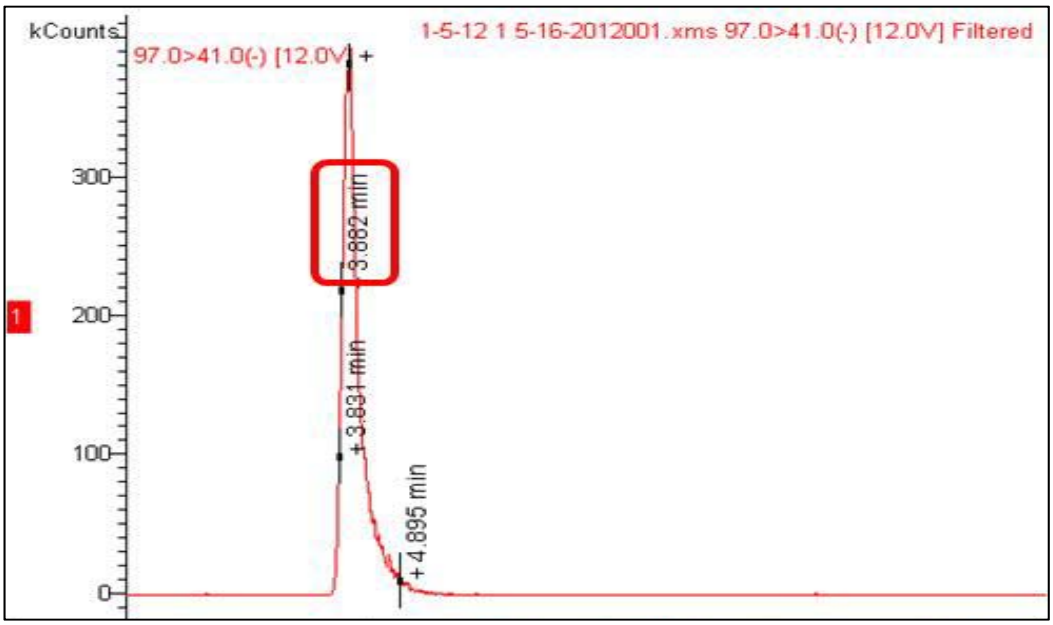


Figure 2. LC-MS/MS chromatogram of a maize sample (t_R MON = 3.9 min).

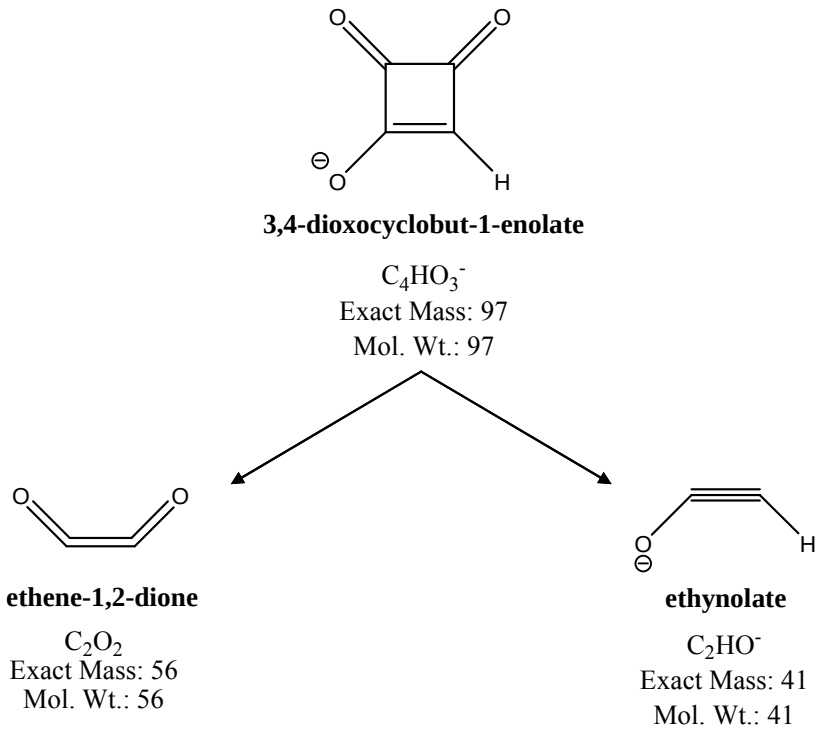


Figure 3. Presumed MON fragmentation pathway.